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Comparison of the Growth and Survival of Larval Turbot in the Absence of Culturable Bacteria with Those in the Presence of *Vibrio anguillarum*, *Vibrio alginolyticus*, or a Marine *Aeromonas* sp.

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Larval turbot (*Scophthalmus maximus*) were reared on rotifers (*Brachionus plicatilis*) in the absence of culturable bacteria for up to 14 days and exhibited growth and high rates of survival (>55% in five experiments). Low numbers of known bacteria were introduced into similar cultures by exposure of the rotifers to a suspension of bacteria prior to addition of rotifers to the larval cultures; *Vibrio anguillarum* 91079 caused a highly significant decrease ($P < 0.01$) in the proportion of survivors in two separate trials. With an *Aeromonas* sp. previously isolated from a healthy batch of copepod-fed larvae, there was no significant difference in survival compared with control larvae, even though the density of bacteria in the water of larval cultures reached 10^7 ml^{-1}. Bacteria colonized the gut of larvae exposed to *Aeromonas*-treated rotifers to levels similar to those in conventionally reared fish (>4×10^4 CFU per larva). Rearing of larvae in the presence of known bacteria provides a means of investigating the interaction of specific bacteria with turbot larvae and could provide a method for the selection of bacteria which may restrict the growth of opportunistic pathogens which would be harmful to turbot larvae.

Turbot (*Scophthalmus maximus*) has been identified as the most suitable species of flatfish for rearing in aquaculture in Northern Europe because of its ease of growth and high market value (16). In the North Sea, wild larvae hatch in inshore waters <40 m in depth and feed on a diet of planktonic cladocerans and copepods (8). In aquaculture, the more easily cultured rotifers, such as *Brachionus plicatilis*, are used for first feeding for 8 to 10 days, when *Artemia* nauplii are substituted. With such rearing systems, intensive culture of turbot is now a well-established process that produces several hundred tons of turbot per year in Europe (17), although the survival rate of larvae during the early rearing stages is still very variable and larval rates of mortality may be extremely high. That bacteria are involved in larval rearing losses is shown by the beneficial effect of antibiotic treatment (4, 5, 15) and the reversion to heavy losses when antibiotic-resistant bacteria develop. Study of the bacterial flora of larval turbot (9, 10, 13) has shown that within 3 to 4 days of first feeding, the number of gut microflora of larvae reached 10^4 to 5×10^5 CFU per larva for various food sources and larval rearing conditions. The development of a significant number of flora coincided with the commencement of feeding by larvae on rotifers or copepods, and the bacteria associated with these food organisms established the gut flora (9, 10, 13), rather than those present in the tank water or associated with the eggs. Pathogenic bacteria have rarely been associated with mortalities in larval fish (9–13), and no correlation between larval survival rates and the presence of recognized bacterial pathogens has been found (10). Thus, the reasons for losses caused by bacteria are poorly understood, and as part of an investigation of the role of bacteria in larval rearing, we have raised larvae in the absence of culturable bacteria (18) so that we may study the effects of specific bacteria introduced into the larval rearing system.

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MATERIALS AND METHODS

Bacteria. *Vibrio anguillarum* 91079, originally isolated from a case of vibriosis in juvenile turbot, was supplied by M. Horne (7); *Vibrio alginolyticus* AR was isolated from larval turbot at Merexo, Spain, and was supplied by A. Riaza. *Aeromonas* sp. strain C39 was isolated from the gut flora of healthy turbot larvae fed on a diet of copepods at Hunterston, Scotland (9).

Algae and rotifers. An axenic culture of *Pavlova lutheri*, supplied by M. Scott, Dunstaffnage Marine Laboratory, Oban, Scotland, was cultured in 2-liter spherical flasks of S88 medium (19) in a continuous culture system as described by Droop (3), except that an Atlantis air pump and 0.2-μm-pore-diameter Sartofluor II air filter (Sartorius) were used for culture aeration instead of a vacuum system.

Bacterium-free cultures of the rotifer *B. plicatilis* were obtained by removal of eggs from adult rotifers and treatment of the eggs in antibiotic solution (oxolinic acid, 10 μg ml^{-1}; kanamycin, 10 μg ml^{-1}; erythromycin, 10 μg ml^{-1}; penicillin G, 150 μg ml^{-1}; streptomycin, 75 μg ml^{-1}) for 24 h before being rinsed in sterile seawater (SW) at 25‰ salinity. The rotifers were cultured in a continuous system (3) in 2-liter spherical flasks of SW at 25‰ salinity supplemented with vitamin B_{12} (1.0 ml of a 0.1-mg liter^{-1} stock solution liter of SW^{-1}) and supplied with axenic *P. lutheri*. As a routine bacteriological sterility check, 25-ml samples of the rotifer or algal cultures were added to 50 ml of brain heart infusion broth (Oxoid) supplemented with 1% NaCl; sterility test broth (Difco) and marine broth (Difco) were similarly inoculated in several initial experiments, but NaCl-supplemented brain heart infusion broth was satisfactory for detection of contaminating bacteria. Broths were incubated for up to 14 days at 20°C and inspected for signs of bacterial growth. Rotifers were also treated with 4′,6-diamidino-2-phenylindole and viewed with a fluorescence microscope to confirm the absence of associated bacteria (6).

Colonization of rotifers with bacteria. Bacteria were cultured in 50 ml of marine broth (Difco) in dimpled, conical flasks with shaking overnight. The bacteria were harvested by centrifugation and rinsed with sterile SW and then were resuspended in sterile SW to a cell density of approximately 1.0×10^9 CFU ml^{-1} (optical density at 600 nm, 1.0). Rotifers were siphoned off from the growth vessel of the chemostat into 250-ml conical flasks for feeding to the turbot larvae from day 3 posthatch. Bacteria were added to give a final cell density of 1.0×10^6 CFU ml^{-1} and incubated with the rotifers for 60 min at 20°C. Enough rotifers were added to each larval rearing flask to give between 1.0 and 3.0 rotifers ml^{-1} (approximately 50 ml of rotifer culture per flask). Inoculated rotifers were only

TABLE 1. Effect of specific bacteria on survival of turbot larvae

Expt	Length of expt (days)	Flask	Bacteria added	Surviving fish [no. of survivors/initial no. (%)]	Significance (χ^2 test)[a]	Length of larvae (mm [mean ± 95% CL])[b]	Bacterial density (CFU ml^{-1})	
							In larvae	In water[c]
1	12	1	*Aeromonas* sp. strain C39	32/35 (91.4)	3.67 (NS)	4.51 ± 0.26	4×10^4	2×10^6
		2	*Aeromonas* sp. strain C39	26/33 (78.8)	0.13 (NS)	5.12 ± 0.16	3.44×10^4	1.5×10^6
		3	None	22/36 (61.1)		ND[d]	ND	ND
		4	None	30/35 (85.7)		ND	ND	ND
		5	None (contaminated)	25/30 (83.3)		5.14 ± 0.08	6.7×10^3	4.1×10^5
		6	None (contaminated)	32/40 (80.0)		5.09 ± 0.30	3×10^2	1.3×10^5
		7	None (contaminated)	32/36 (88.9)		5.37 ± 0.15	4×10^3	1.0×10^6
2	14	1	*Aeromonas* sp. strain C39	8/8 (100)	0.91 (NS)	5.14 ± 0.21	3.7×10^4	1.4×10^7
		2	*Aeromonas* sp. strain C39	12/15 (80.0)	<0.1 (NS)	4.68 ± 0.24	1.79×10^4	1.2×10^7
		3	None	5/7 (71.4)		5.17 ± 0.40	<10[e]	<10[e]
3	14	1	*V. anguillarum* 91079	6/26 (23.0)	16.7 (HS)	ND	ND	1.6×10^5
		2	*V. anguillarum* 91079	0/21 (0)	29.7 (HS)	ND	ND	1.1×10^5
		3	None	27/34 (79.4)		ND	ND	<10[e]
4	14	1	*V. anguillarum* 91079	7/47 (14.9)	7.0 (HS)	ND	ND	2.7×10^5
		2	*V. anguillarum* 91079	4/48 (8.3)	12.3 (HS)	ND	ND	2.9×10^5
		3	None	16/37 (43.2)		ND	ND	<10[e]
5	14	1	*V. alginolyticus* AR	13/36 (36.1)	0.8 (NS)	5.01 ± 0.30	1.75×10^5	5×10^6
		2	*V. alginolyticus* AR	9/33 (27.3)	<0.1 (NS)	5.02 ± 0.66	4×10^4	4.7×10^4
		3	None	7/29 (24.1)			<10[e]	<10[e]
		4	None	8/30 (26.7)			<10[e]	<10[e]

[a] For χ^2 tests, the survival rates in flasks with added bacteria were compared with those in control flasks without bacteria. HS, highly significant, $P < 0.01$; NS, not significant, $P > 0.1$.
[b] CL, confidence limit.
[c] Maximum density reached during the experiment.
[d] ND, not done.
[e] No bacteria detectable.

fed to larvae on the first day of feeding. The density of rotifers was maintained at 2.0 to 3.0 rotifers ml^{-1} thereafter by the daily addition of axenic rotifers.

Turbot larvae. To eliminate culturable bacteria from the larvae, the procedure of Scott and Barbour (18) was followed. Fertilized turbot eggs (2) were suspended in antibiotic solution (as for bacterium-free rotifers) for 24 h at 15°C. The eggs were then removed from the antibiotic solution, rinsed in sterile SW at 25‰ salinity, and allowed to hatch in 5-liter spherical flasks (40 eggs per flask) filled with full-strength (32‰ salinity) SW. Prior to addition of the turbot eggs, axenic *P. lutheri* was added to the rearing flasks to create a "green-water" system. It has been suggested (1) that the improvement in growth of juvenile sole in the presence of unicellular algae was due to reduction of the dissolved ammonia levels by the algae. The nutritional quality of the rotifers is also maintained by the presence of algae in the larval rearing water. Once the eggs hatched, a low level of filtered (0.2-μm pore diameter) air was supplied through airstones, and the temperature was gradually increased from 15 to 20°C over a period of 4 or 5 days. Twenty-five-milliliter samples were removed daily, inoculated into brain heart infusion broth–1% NaCl, and tested for the presence of culturable bacteria as described for algae and rotifers.

Sampling of the bacterial flora of the gut of turbot larvae. Because the small size of turbot larvae rendered dissection impractical, the aerobic bacterial flora of the intestine was sampled by a method similar to that of Muroga et al. (11). Turbot larvae (10 fish per sample) were placed in sterile glass tubes (internal diameter, 38 mm) with nylon mesh (10 μm pore diameter) fixed to the bottom. The larvae were anesthetized by immersion in 0.1% (wt/vol) benzocaine in SW for 30 s and then rinsed with 0.1% (wt/vol) benzalkonium chloride in SW for 60 s, followed by sterile SW only for 60 s, to remove the surface bacteria. The larvae were aseptically transferred to a sterile glass homogenizer (Jencons) and homogenized in 1.0 or 2.0 ml of sterile SW. Dilutions were prepared in sterile SW, and 0.1-ml volumes were spread onto marine agar 2216 (Difco) plates. The plates were incubated at 20°C for 5 days before the bacterial colonies were counted.

RESULTS

Effect of *Aeromonas* sp. strain C39 on survival of turbot larvae. Of the seven flask cultures in experiment 1, three were contaminated with a gram-negative, yellow-pigmented bacterium before first feeding began on day 3, but these cultures were retained so that the effect of this bacterium on larvae could also be monitored. Axenic rotifers were introduced into flasks 3 to 7 (Table 1), and rotifers colonized with *Aeromonas* sp. strain C39 were added to flask cultures 1 and 2. On termination of the experiment after 12 days, rates of survival of larvae in the flasks ranged from 61.1 to 91.4%, the lowest value being found in a control flask (Table 1, experiment 1, flask 3), although there was no significant difference in the rates of survival between the control culture and the other cultures. Colonization of rotifers with *Aeromonas* sp. strain C39 appeared to have no deleterious effect on larvae (Table 1), with colonization of the larval gut to normal levels (3.4×10^4 to 4×10^4 CFU per larva) and a high rate of survival, despite the presence of high levels of bacteria in the water for several days (Table 1 and Fig. 1). In the flasks which were contaminated, the larvae were also unaffected, with a high rate of survival but with a lower level of colonizing bacteria in the gut (Table 1).

A second experiment with *Aeromonas* sp. strain C39-colonized rotifers and a lower density of larvae gave similar results, with no significant difference in survival between control and bacterium-containing cultures (Table 1, experiment 2). The density of *Aeromonas* sp. strain C39 cells was approximately 10^7 ml^{-1} in both flasks for the final 3 days of the experiment, and the density of gut bacteria was similar to that in conventionally reared larvae (>10^4 per larva [Table 1]).

Effect of *V. anguillarum* on survival of larvae. Because *Aeromonas* sp. strain C39, which was isolated from a batch of larval turbot showing a high rate of survival when fed on copepods, had no deleterious effect on larvae in the experiments described above, despite growing to a very high bacterial cell density in water, the effect of a recognized pathogen was in-

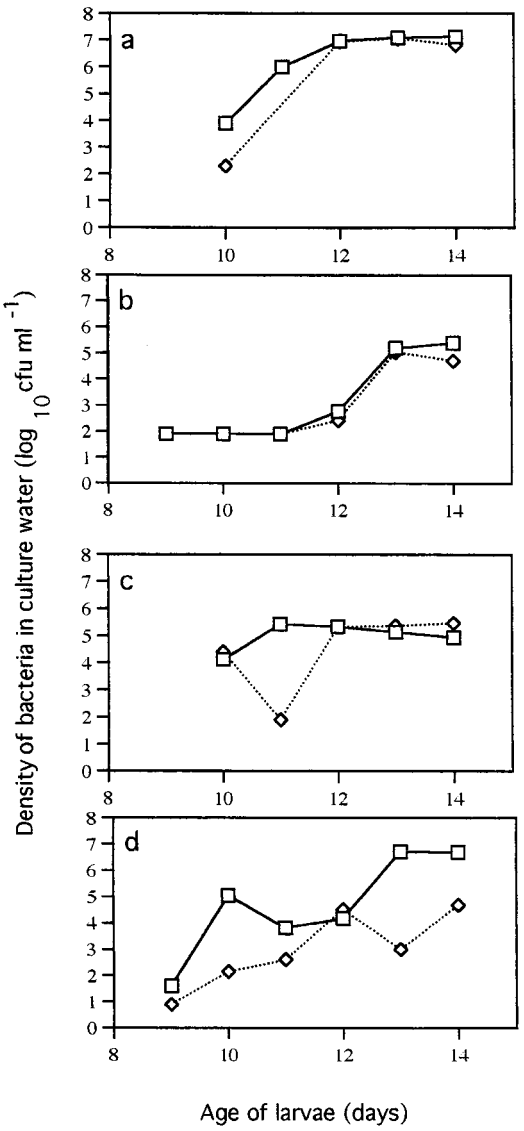

FIG. 1. Concentration of bacteria in the water of cultures of turbot larvae fed on rotifers colonized with specific bacteria. The bacteria used to colonize rotifers were *Aeromonas* sp. strain C39, experiment 2 (a); *V. anguillarum* 91079, experiment 3 (b); *V. anguillarum* 91079, experiment 4 (c); *V. alginolyticus* AR, experiment 5 (d). □, flask 1; ◇, flask 2. Experiment and flask numbers are shown in Table 1.

vestigated. *V. anguillarum* 91079, isolated from an outbreak of vibriosis in juvenile turbot at Golden Sea Produce, Hunterston, Scotland (7), was used to colonize rotifers in experiments 3 and 4 (Table 1). In both experiments, the cultures containing bacteria had low rates of survival and the differences in survival, compared with control cultures, were highly significant (Table 1). The cell density of *V. anguillarum* in the culture water followed a different course from that of cultures containing *Aeromonas* sp. strain C39, being $<10^2$ CFU ml^{-1} until day 12 in experiment 3 (Table 1) and reaching maximum cell densities which were 1/10th to 1/100th of those with *Aeromonas* sp. strain C39 (Table 1). Because so few larvae from the test groups survived the experiment, no data about growth rates were obtained.

Effect of a *V. alginolyticus* field isolate on larval survival. An isolate identified as *V. alginolyticus*, obtained from a commercial turbot hatchery and thought to be associated with larval mortalities, was used to colonize rotifers in experiment 5. Although the rates of survival in control flasks (24.1 and 26.7%) were lower than those in previous experiments, there was no significant difference in the rate of survival in the presence of *V. alginolyticus* AR (Table 1), and bacterial densities in the larval gut and in culture water were similar to those of conventional larval cultures.

DISCUSSION

In the five rearing experiments reported here, larvae hatched from disinfected eggs were allowed to develop for 3 days before addition of either axenic or bacterially colonized rotifers. Because contamination could have resulted from incomplete decontamination of a single one of the >30 eggs usually added to each flask, it was necessary to initiate up to eight flask cultures and to discard any which subsequently showed contamination. However, it was important to avoid prolonged exposure of eggs to antibiotics, because this was damaging to larval growth, development, and survival (data not shown). Even when contamination of flasks did occur, this did not necessarily lead to a low rate of survival. In experiment 1 (flasks 5 to 7), a high rate of survival was found, but the level of colonization of larvae by the contaminating bacteria was lower than those in other experiments, perhaps because the bacteria did not colonize rotifers efficiently and thus were not introduced into the gut in large numbers. The extremely high rate of survival attainable in the absence of bacteria is further evidence for the role of bacteria in larval mortality. However, the numbers, nature, and pathogenic mechanisms of these bacteria are not known; few putative pathogens have been isolated from larvae (10), and it would be necessary to test such bacteria individually to confirm their virulence.

A further conclusion from the high rate of survival in control flasks which remained free of detectable bacterial contamination is that bacteria are not essential for survival, nutrition, or triggering of developmental responses in the larval turbot. Although it is not practical to develop a bacterium-free rearing system for use under farm conditions, the results show that control of the types of bacteria present, rather than the total density, may be important for attaining high rates of survival.

Introduction of *Aeromonas* sp. strain C39 into the culture system led to concentrations of this bacterium in the water which would normally be considered harmful in conventional rearing trials, but larvae were not visibly affected. Because the aeromonad was isolated from a batch of larvae which had a high rate of survival, this result was not unexpected, as was the demonstration that the pathogen *V. anguillarum* 91079 caused high rates of mortality in the cultures. Whereas the mean survival rates of larvae with *V. anguillarum* 91079 were 14.5 and 26.9% of those of the control cultures in experiments 3 and 4, respectively, the mean survival rates in cultures containing other bacteria were higher than those in the relevant bacterium-free control cultures (15.9% greater, experiment 1, C39; 14.4% greater, experiment 1, contaminants; 26.0% greater, experiment 2; and 24.8% greater, experiment 5). Although individually these differences were not statistically significant, one possible reason for the higher rate of survival of larvae in cultures containing bacteria (except for *V. anguillarum*) compared with bacterium-free cultures could be due to reduction of the ammonia concentration in cultures, because this was higher in bacterium-free cultures (results not shown) and ammonia is readily utilized by heterotrophic marine bacteria (21).

The bacteria tested here represent the extremes of larvae-bacterium interactions, and the assay could be used to test a

range of bacteria to establish their possible beneficial effect in larval rearing. The establishment of a stable gut microflora is important in the health and digestive function of animals (20), and a resident microflora may be established in fish (14). To test large numbers of bacteria in the present system would be laborious, but the demonstration that larvae develop and grow normally in the presence of high concentrations of the appropriate bacteria indicates that a search for bacteria which could be of benefit in larval rearing would be worthwhile.

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